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INTRODUCTION

The hemostatic system plays an important role in the regulation of tumor angiogenesis and progression. Prostate cancer is one of most common cancers affecting American men. The initiation and progression of prostate cancer remain not well understood to enable rational development of interventional therapy. In this proposal, we propose to study thromboxane synthase and its product, thromboxane A_2 , in prostate cancer progression. Thromboxane synthase is an enzyme downstream cyclooxygenase, utilizing prostaglandin H to form thromboxane A_2 . Thromboxane A_2 is a potent inducer of platelet aggregation which can subsequently lead to coagulation and thrombosis, a hematological complication affecting about 15% to 20% of cancer patients and the second leading cause of death in cancer patients. Platelet aggregation also release a plethora of angiogenesis regulators and fibrin deposition to facilitate angiogenesis and formation of tumor stroma. In addition, it has been demonstrated that thromboxane A_2 also can directly modulate endothelial cell angiogenic responses. It is our working hypothesis that TXA₂ may play an important role in prostate tumor progression and that this functional role of TXA₂ is achieved by modulating tumor cell motility, endothelial angiogenic responses, and platelet aggregation. TXA₂ produced by PCa cells can promote PCa cell migration as an autocoid and modulate endothelial cell angiogenic responses and stimulate platelet aggregation as a paracrine factor. Platelet aggregation release various angiogenesis regulators and cause coagulation, which subsequently leads to deposition of fibrin at tumor sites. These events, collectively, promote tumor angiogenesis and growth. The proposed work will provide significant insights into how prostate cancer cells regulate cell migration and hemostatic system to facilitate tumor angiogenesis, growth, and metastasis. The knowledge obtained from the proposed work will identify key targets (TX synthase and TXA₂ receptor) to develop interventional therapy for prostate cancer, advancing the program's eventual goal to eliminate prostate cancer.

BODY OF REPORT

List of Technical Objectives

1. Perform a correlation study in 200 cases of prostate cancer patients to evaluate correlation between TX synthase expression in prostate tumor tissues and their grade and stage.
2. Study the effects of increased TX synthase expression on cell migration, proliferation, and apoptosis in DU145 cells.
3. Study whether TX synthase-overexpressing DU145 cells have increased ability to induce platelet aggregation in vitro and in vivo.
4. Evaluate the growth rate of s.c. tumors derived from TX-synthase overexpressing DU145 cells and compare with that of control DU145 cells.
5. Evaluate whether there is any difference, as a result of TX synthase expression, in tumor growth and spontaneous metastasis in an orthotopical model.
6. Evaluate whether TX synthase inhibitor CI and TXA2 receptor antagonist, SQ29,548, inhibit PCa tumor growth.

Research Progress

Objective 1. Perform a correlation study in 200 cases of prostate cancer patients to evaluate correlation between TX synthase expression in prostate tumor tissues and their grade and stage.

This technical objective has been achieved. Further we have cloned, sequenced, and characterized full-length TX synthase cDNA. Here we report the following findings:

1). *Immunohistochemical analysis.* We have analyzed the expression of TX synthase in about 200 cases of human CaP specimens in three independent studies. The staining pattern for TxS was associated with staining intensity. When the cells were weakly or moderately stained, immunoreactivity was mainly confined to the supra-nuclear region. When the cells were strongly stained, immunoreactivity became diffusely cytoplasmic. TX synthase was strongly expressed in the complex papillary cribriform area (Gleason pattern 4) but only weakly in the well-formed glands (Gleason pattern 3) (Fig. 1 A). In well-formed glands, strong expression of TX synthase was detected at the most invasive edges including perineural and capsular invasions (Fig. 1B).

In normal prostatic glands, TxS immunoreactivity was either weak or not detected in the secretory cells and was weak to moderate in the basal cells. Increased TxS expression (with intensity 2 or 3) was mainly observed in the areas of inflammation and atrophy in the benign glands. In the glands with high-grade prostatic intraepithelial neoplasia (HGPIN), TxS expression was uniformly increased with intensity ranging between 2 and 3. A total of 38 of 40 prostatic carcinomas (95 %) were moderately and strongly stained for TxS focally or diffusely. The H-score of TxS expression in prostatic carcinoma ranged from 100 to 280 (median 200). The H-score was significantly associated with tumor differentiation and tumor extent (Table 1). High expression (H-score ≥ 200) was observed in 11 of 23 well to moderately differentiated tumors (Gleason score $\leq 3+4$) (48%) and 14 of 17 moderately to poorly differentiated tumors (Gleason score $\geq 4+3$) (82%) correspondingly ($p=0.026$). In the organ-confined or advanced tumors, a high H-score (≥ 200) was found in 7 of 19 (37%) or 18 of 21 (86%) cases respectively ($p=0.001$). There was no significant difference in H-scores between two age groups. In addition, a few poorly to undifferentiated tumor foci (Gleason pattern 5) showed reduced TxS expression. No immunoreactivity was observed in the sections where the primary antibody was omitted (negative controls).

Perineural invasion (or growth of tumor cells along nerve sheaths) is a common finding in prostatic carcinoma. Extensive perineural invasions are often associated with tumor extension into extraprostatic soft tissue²⁹. In this study, 24 of 40 (60%) cases showed evident perineural invasions. Remarkably, in over 90% of the perineural invasions, neoplastic glands were diffusely and intensely stained for TxS.

A.



B.

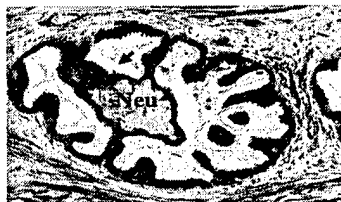
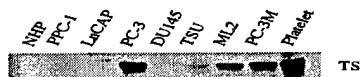


Figure 1. Immunohistochemical analysis of TX synthase expression in 2 representative human CaP tissue specimens. Brown to reddish color indicates positive staining. **A.** Photomicrograph of prostatic adenocarcinoma with strong cytoplasmic expression of TX synthase in GS 4 (major) area but not in GS 3 (major) area. **B.** Association of TX synthase expression with perineural invasion as indicated by the arrow.

2). *Expression and Cloning of TX synthase.* Western Blot analysis revealed that PC-3, PC-3M, and ML-2 cells expressed much higher level of TX synthase than did normal prostate epithelial cells or other tumor cell lines such as DU145 and LNCaP cells (**Fig. 2 A**). RT-PCR analysis using TX synthase specific primers revealed that the level of TX synthase mRNA was much higher in PC-3 cells than in DU145 (**Fig. 2 B**). We cloned the whole open reading frame of TX synthase from PC-3 cells into a pcDNA3.1 expression vector. Transient transfection of DU145 cells with the construct greatly increased the level of TX synthase in DU145 cells, suggesting the TX synthase cDNA was correctly inserted into pcDNA expression vector (**Figure 2 D**). Using the cloned TX synthase cDNA as a probe, we found a strong expression of TX synthase mRNA in PC-3 cells as compared to DU145 or LNCaP cells (**Fig. 2 C**).

A



B.



C: Northern Blot



1. RNA Standards; 2. DU145; 3. HEL;
4. LNCaP; 5. PC-3

Figure 2. Expression of TX synthase in different CaP cell lines. **A.** Immunoblot analysis. Platelet lysates as positive control. **B.** RT-PCR analysis. Shown here is one round PCR product using TX synthase specific primers. **C.** Northern blot analysis. Note the much higher expression of TX synthase expression in PC-3 than DU145 and LNCaP.

In a cancer profiling array which contained three sets of cDNA from matched normal and tumor prostate tissues, we found two out of three cases had elevated expression of TX synthase in tumor tissues when compared to the matched normal tissue (Data not shown).

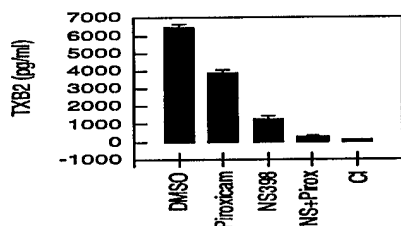


Figure 3. Biosynthesis of TXA₂ is dependent on COX activity.

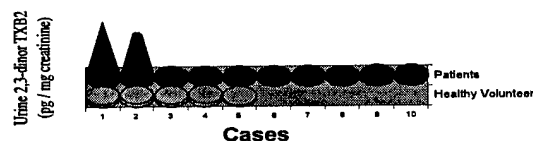


Figure 4. Marked increase in TX synthase activities in two CaP patients.

3). *Biosynthesis of TXA₂ in CaP cells: its relationship with COX.* TxS utilizes intermediate prostanoid product of COX to form TXA₂, a bioactive eicosanoid involved in platelet activation and aggregation, vessel constriction, and proliferation of smooth muscle cells. We hypothesize that TxS may contribute to PCa progression through the activities of TXA₂. We evaluated whether TXA₂ is produced in PCa cells especially when we found that there were two variations in the amino acid sequence of TxS expressed in PC-3 cells. First we compared the biosynthesis of TXA₂ in PC-3 cells with that in HEL cells, an erythroleukemia cell line²⁴, by measuring the accumulation of TXB₂, the stable product of TXA₂, in culture supernatants. The concentration of TXB₂ in culture supernatants from PC-3 cells was 1295 pg/ml, compared to 580 pg/ml in culture supernatants from a same number of HEL cells. Treatment with CI (10 μM), a TxS inhibitor, reduced the production of TXA₂ from 1295 pg/ml to 685 pg/ml in PC-3 cells. At the same concentration, CI also reduced the production of TXA₂ from 580 pg/ml to 292 pg / ml in HEL cells. The results suggest that TxS expressed in PC-3 cells were active enzymatically, even though it had two variations in amino acid sequence (**data not shown**). The results further suggest that PC-3 cells produced more TXB₂ than HEL cells. More extensive studies are needed to fully evaluate whether the higher level of TXB₂ biosynthesis in PC-3 cells, compared to HEL cells, is due to the two mutations in TxS or a dysregulation of TxS activity in PC-3 cells.

As an enzyme downstream of COX, TxS converts PGH₂ to TXB₂. Expression of COX-2 and COX-1 in prostate cancer has been extensively reported³⁻⁷. Through Western blot, we also detected the expression of both COX-1 and COX-2 in PC-3 cells (data not shown). To study whether TXA₂ production is dependent on COX activities, PC-3 cells were treated with either 50 μM piroxicam, a COX-1 selective inhibitor (IC₅₀ for COX-1 is 17.7 μM, for COX-2, over 500 μM)²³, or 10 μM NS398, a highly selective inhibitor for COX-2 with IC₅₀ of 1 μM²³⁻²⁵, or a combination of the two inhibitors. As shown in **figure 3**, piroxicam alone reduced TXA₂ synthesis by approximately 40%. Treatment of PC-3 cells with NS398 reduced TXA₂ production by ~ 80%. Treatment of PC-3 cells with both piroxicam and NS398 reduced TXA₂ production by 95%, a level comparable to that achieved by the TxS inhibitor, CI. The data suggest that both COX-1 and COX-2 can provide TxS with the substrate, PGH₂, for TXA₂ synthesis.

4). *Marked increase in TXA₂/TXB₂ levels in some prostate cancer patients.* The stable TX product from TX synthase activity, TXB₂, can be further converted into 2,3-dinor TXB₂ and secreted in urine. The level of urine 2,3-dinor TXB₂ has been used as a surrogate marker for TX synthase activities in the human body. We measured the levels of urine 2,3-dinor TXB₂ in 10 prostate cancer patients and 5 healthy volunteers. As shown in the **figure 4**, two patients had a dramatic increase (15 ~ 20 folds) in TXB₂ levels when compared with other patients and healthy volunteers. While a study with larger population is needed for any potential diagnostic application, the data do suggest at least a subset of CaP patient population (~ 20%) have markedly increased TX synthase activities.

Objective 2 Study the effects of increased TX synthase expression on cell migration, proliferation, and apoptosis in DU145 cells. This objective has been largely achieved as described below.

1). *Stimulation of DU145 cell migration by increased expression of TX synthase.* Transient transfection of DU145 cells with a TX synthase expression construct (Echo 6, Fig. 5) increased the steady-state level of TX synthase in DU145 cells. Increased expression of TX synthase in DU145 cells stimulated cell migration on fibronectin (Fig. 6).

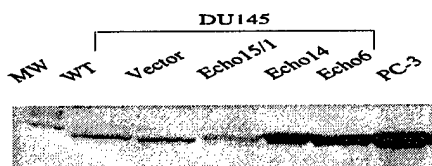


Figure 5. Transfection of DU145 cells with TX synthase expression constructs increased the steady state level of TX synthase protein.

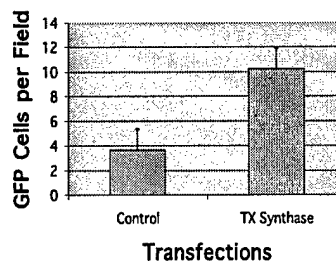


Figure 6. Stimulation of DU145 cell migration by transient transfection of DU145 cells with TX synthase expression construct.

2). *TX synthase activity is involved in cell migration on fibronectin.* As shown in **Fig. 7**, CI, a TX synthase inhibitor, dose-dependently inhibited PC-3 cell migration on fibronectin. Another TX synthase inhibitor, fureglerate sodium, had similar effect (Data not shown). A TXA_2 receptor antagonist, SQ29,548, at 10 μM significantly reduced PC-3 migration (**Figure 8**) while U46619, a TXA_2 receptor agonist, stimulated PC-3 migration (**Fig. 9**). These findings suggest a role of TXA_2 in modulation of CaP cell motility.

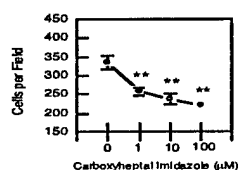


Figure 7. Inhibition of PC-3 Cell Migration on Fibronectin by TX synthase Inhibitor CI. **, $P < 0.01$ when compared to vehicle control.

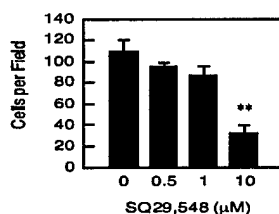


Figure 8. Inhibition of PC-3 migration by SQ29548. **, $P < 0.01$ when compared to vehicle control.

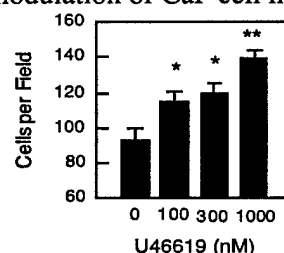


Figure 9. Stimulation of PC-3 Migration by U46619. *, $P < 0.05$ and **, $P < 0.01$ when compared to the control.

Objective 3. Study whether TX synthase-overexpressing DU145 cells have increased ability to induce platelet aggregation *in vitro* and *in vivo*.

This objective is completed.

Using washed expired platelets from blood bank, we found U46619 and thrombin can induce platelet aggregation and release of VEGF (**figure 10**). Interestingly, PC-3 cells also caused platelet aggregation and release of VEGF. Pretreatment of PC-3 cells with a TX synthase inhibitor, CI, reduced the release of VEGF (**figure 13**). We are now testing whether DU145 cells also can cause platelet aggregation.

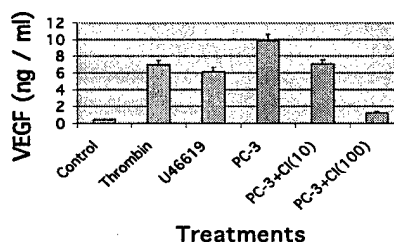


Figure 10. Induction of platelet aggregation and VEGF release by PC-3 cells: Effect of CI.

Objective 4. Evaluate the growth rate of s.c. tumors derived from TX-synthase overexpressing DU145 cells and compare with that of control DU145 cells.

The studies proposed in this specific aim have been completed.

We have injected TX-synthase overexpressing DU145 cells and their control into athymic nu/nu mice and found no significant difference in the growth of tumors. One major compounding factor is the endogenous expression of TX synthase, albeit at low level. Therefore

Objective 5. Evaluate whether there is any difference, as a result of TX synthase expression, in tumor growth and spontaneous metastasis in an orthotopical model.

This objective has been achieved.

When PC-3 cells were orthotopically implanted into mouse prostate, spontaneous metastasis to adjacent tissues and lymph nodes sometimes occurs. We examined the level of TX synthase and its activity in cells isolated from orthotopic tumors (PC-3/PI) and in those from lymph metastasis (PC-3/PI-Ln). As shown in **Fig. 11**, there was an increase in TX synthase protein levels in PC-3/PI-Ln than in PC-3/PI. PC-3/PI-Ln cells also produced about 50% more TXA₂ than did PC-3/PI cells (**Fig. 11B**). When plated on fibronectin, increased cell migration was observed with PC-3/PI-Ln, as compared to PC-3/PI (**Fig. 11C**), suggesting that increased TX synthase level increases cell motility.

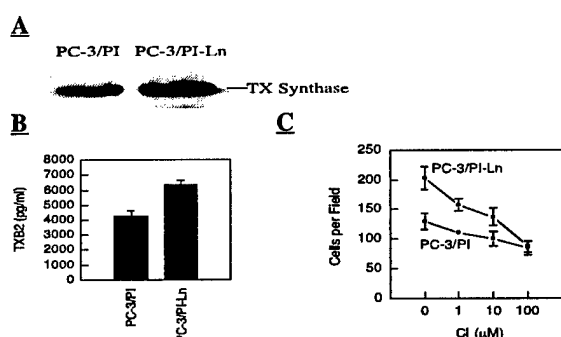


Figure 11. Increased TX synthase expression (A), activity (B), and motility (C) in PC-3 cells (PC-3/PI-Ln) isolated from lymph node metastasis, when compared to PC-3 cells in primary tumor (PC-3/PI).

Objective 6. Evaluate whether TX synthase inhibitor CI and TXA₂ receptor antagonist, SQ29,548, inhibit PCa tumor growth.

This objective has been achieved.

It has been shown that COX inhibitors such as Celecoxib can induce apoptosis in CaP cells (Hsu et al., 2000) and also various other tumor cells. Since TX synthase is an enzyme downstream of COX, we studied the effect of PTA₂, a dual inhibitor of TX synthase and TXA₂ receptor functions, on CaP cell survival. As shown in the **figure 12**, PTA₂ induced PC-3 cell apoptosis at concentration of 5 μM. When treated with PTA₂, there was increased expression of tumor suppressor Cip-1/WAF-1, decreased expression of Bcl-2 and cyclin B (**Figure 13**).

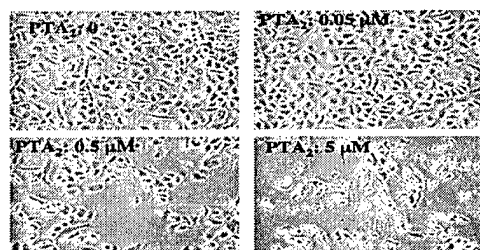


Figure 12. Morphology of PC-3 cells 24 hours after treatment with PTA₂ (phase contrast). Note the induction of PTA₂ at 5 μM concentration.

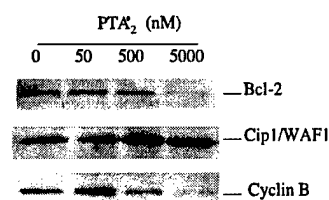


Figure 13. Effect of PTA₂ on the expression of Bcl2, Cip1/WAF1, and cyclin B in PC-3 cells.

To study the role of TX synthase in PCa progression, PC-3 cells, premixed with a 0.4 ml of aliquote of Matrigel, were injected s.c. into the right flank of athymic nude mice and TX inhibitor CI (20 mg/kg mouse weight) were injected into the implants once a week. The use of Matrigel was to promote the growth of tumors from PC-3 cells (Pretlow et al., 1991) and to serve as a depot for administering CI, a TX synthase

inhibitor. The use of intra-gel injection was to employ limited amount of CI to demonstrate the effect of TX synthase inhibition on tumor growth. As shown in **Figure 14 A**, CI reduced the growth of tumors derived from PC-3 cells and tumor weight (**Figure 14 B**). **Figure 14 C** shows a representative tumor from the control and CI-treated groups. Note the accumulation of blood and clotting (arrowed) on the surface of control tumors, as compared to the CI-treated group. In the control tumor, blood accumulation and clot are obvious on the surface of tumors. In contrast, in CI-treated implant, blood accumulation and angiogenesis on the surface angiogenesis are significantly reduced. Histological analysis indicated that, in the tumors without CI treatment, across the whole sections were tumor cells. In contrast, in the groups treated with CI, tumor cells were distributed as small islands, suggesting an inability to expand continuously. The reduction of tumor growth by CI may be related to its direct inhibition of endothelial cell angiogenic responses (Nie et al., 2000). Another possibility is that inhibition of TX synthase activity in PC-3 cells disrupted the interaction between PC-3 cells with host tissues, such as angiogenesis, that may turn out critical for tumor growth.

Inhibition of Tumor Growth by Topical Administration of CI

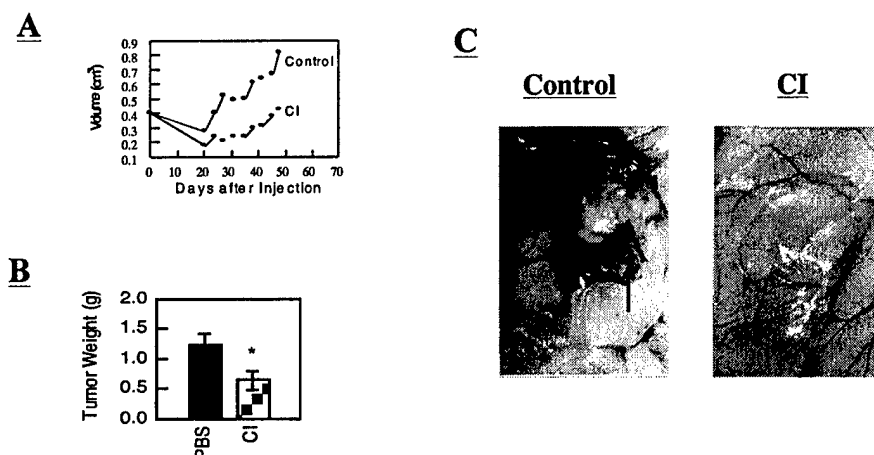


Figure 14. Inhibition of prostate tumor growth by TX synthase inhibitor CI. **A.** Growth kinetics of tumors in the presence of CI. n=4 for control; n=8 for CI. **B.** Gross morphology of tumors. Note the accumulation of blood and clotting (arrowed) on the surface of control tumors. In contrast, minimal vessel penetration toward CI-treated group. **C.** Comparison of tumor weights. *, p < 0.05.

Discussion and Conclusion:

Our findings suggest that TX synthase is expressed in prostatic adenocarcinoma tissues, especially those cells at the edge of tumor and sites of perineural invasion. In established PCa cell lines, PC-3 cells notably express high levels of TX synthase. TX synthase expressed is enzymatically active and the biosynthesis of TXA₂ is dependent on COX-1 and COX-2 activities. We further found that inhibition of endogenous TXA₂ synthesis by CI significantly reduced PC-3 migration on fibronectin and increased expression of TX synthase in DU145 cells enhance DU145 cell migration. A dual inhibitor of TX synthase and TXA₂ receptor antagonist, pinane thromboxane A₂, was found to cause cell cycle arrest and apoptosis in PC-3 cells. Finally we found that PCa cells can cause platelet aggregation and release of VEGF. Treatment with TX synthase inhibitor, CI, reduced VEGF release. Our findings so far suggest a contributory role of TX synthase in PCa progression.

KEY RESEARCH ACCOMPLISHMENTS

- The expression of TX synthase in prostate cancer specimens was confirmed.
- The level of expression was related to the stage of tumor, perineural invasion and metastasis.
- We cloned full-length TX synthase cDNA from PC-3 cells and constructed an expression construct of tumor cell TX synthase.
- Inhibition of TX synthase was found to reduce cell migration.
- Increased expression of TX synthase stimulated DU145 cell migration.
- PCa cells cause platelet aggregation and the release of VEGF.
- TX synthase inhibitor, CI, can reduce the release of VEGF during platelet aggregation.
- Pinane thromboxane A2, a dual inhibitor of TX synthase and TXA2 receptor antagonist, induced cell apoptosis in PC-3 cells.
- Treatment of prostate tumor with a TX synthase inhibitor, CI, reduced tumor growth and weight. CI treatment also reduced blood coagulation within tumors.

REPORTABLE OUTCOMES

- Article published.
Nie D, Che M, Zacharek, A; Qiao, Y; Cai Y; Tang, K; Lamberti, M; Grignon, D and Honn, KV. Thromboxane Synthase Expression in Prostate Cancer: Involvement in Perineural Invasion. *American Journal of Pathology* 164: 429 – 439, 2004.
- Article to be submitted.
Nie D; Qiao Y; Zacharek, A and Honn, KV. Regulation of prostate cancer metastatic phenotypes by thromboxane A2 through small GTPase Rho. In submission to the *Journal of Biological Chemistry*.
- Abstract published.
Nie, D., Che, M., Honn, K. V. Up-regulation of thromboxane synthase in human prostate carcinoma: Role in tumor progression. *Proc. Amer. Assoc. Cancer Res.* 42: 44, 2001.
- Abstract published.
Nie, D., A. Zacharek, M. Che, Y. Cai, Y. Qiao, M. Lamberti, K. Tang, D. Grignon, and K. V. Honn. Expression, regulation, and function of thromboxane A2 synthase in cancer. *Proc. Amer. Assoc. Cancer Res.* 43: 1, 2002.
- Abstract published.
Nie, D., Y. Qiao, A. Zacharek, and K. V. Honn. Hemostatic regulation of prostate cancer progression: Modulation of tumor cell metastatic phenotypes by thromboxane A2 through activation of Rho. *Proc. Amer. Assoc. Cancer Res.* 43: 9, 2002.
- Review article published.
Nie, D., M. Che, D. Grignon, K. Tang, and K. V. Honn. Role of eicosanoids in prostate cancer progression. *Cancer Metastasis Rev.* 20:195-206, 2001.
- Review article published.
Nie, D. and K.V. Honn. Cyclooxygenase, lipoxygenase, and tumor angiogenesis. *Cell Mol Life Sci.* 59: 799 – 807, 2002.

- Presentation.
Nie, D. and K. V. Honn. "Thromboxane synthase expression in prostate cancer: Role in tumor progression." CapCURE Annual Research Retreat. Lake Tahoe, NV. September, 2001. (Invited presentation).
- Presentation.
Nie, D. "Expression, regulation, and function of thromboxane A₂ synthase in cancer." 7th International Conference on Eicosanoids and Other Bioactive Lipids in Cancer, Inflammation and Related Diseases. Nashville, TN. October 14 – 17, 2001. (Podium Presentation).
- Presentation.
Nie, D., A. Zacharek, M. Che, Y. Cai, Y. Qiao, M. Lamberti, K. Tang, D. Grignon, and K. V. Honn. Expression, regulation, and function of thromboxane A₂ synthase in cancer. 93th Annual Meeting (2002) of American Association for Cancer Research. San Francisco, CA. April 6 - 10, 2002.
- Presentation.
Nie, D., Y. Qiao, A. Zacharek, and K. V. Honn. Hemostatic regulation of prostate cancer progression: Modulation of tumor cell metastatic phenotypes by thromboxane A₂ through activation of Rho. 93th Annual Meeting (2002) of American Association for Cancer Research. San Francisco, CA. April 6 - 10, 2002.
- Patents applied: None.
- Degrees obtained that are supported by this award: None.
- Development of cell lines, tissue or serum repositories: None
- Funding applied or obtained: Yes

Based on the above-described findings, the PI applied for additional funding to initiate translational research on the feasibility of using TX synthase inhibitors or TXA₂ receptor antagonists for the treatment of prostate cancer in 2001. The application was declined.

We also submitted an R01 grant application to NIH, on the basis of above-described findings. The grant is pending.

CONCLUSIONS:

Our studies in the first year of this grant suggest that TX synthase is expressed in human prostate carcinoma and its activity may be involved in the initiation and progression of prostate cancer. As an enzyme downstream cyclooxygenase, TX synthase utilizes prostaglandin H to form thromboxane A₂. Using immunohistochemistry analysis, we found that 25% of clinical prostate tumor specimens had strong expression of thromboxane synthase; 33% of cases had medium expression and 42% of cases had weak expression of thromboxane synthase. Prostate cancer cells isolated from lymph node metastasis had higher levels of thromboxane synthase expression and activity than those isolated from the primary tumor sites in an animal model. We cloned and sequenced full-length thromboxane synthase cDNA from PC-3 cells and constructed an expression vector. Increased expression of thromboxane synthase in DU145 cells was found to stimulate cell migration. Inhibition of thromboxane synthase or blockade of thromboxane A₂ function inhibited prostate cancer cell migration. Therefore, TX synthase and its eicosanoid product, TXA₂, may play a contributory role in prostate cancer progression.